

a1 4. The isolated nucleic acid of claim 1, wherein said nucleic acid comprises a nucleic acid encoding a C domain lacking one or more His residues of the conserved HHxxxDG (SEQ ID NO:4) active site for transpeptidation.--

a8 28. The polypeptide claim 25, wherein said polypeptide comprises a C domain lacking one or more His residues of the conserved HHxxxDG (SEQ ID NO:4) active site for transpeptidation.--

These amendments are made without prejudice and are not to be construed as abandonment of the previously claimed subject matter or agreement with the Examiner's position. In accordance with the requirements of 37 C.F.R. § 1.121, a marked up version showing the changes to the claims, is attached herewith as Appendix A.

REMARKS

Sequence Listing.

This amendment is provided in Response to the Notice to Comply With Requirements for Patent Applications Containing Nucleotide Sequence And/Or Amino Acid Sequence Disclosures. Applicant(s) request entry of this amendment in adherence with 37 C.F.R. §§1.821 to 1.825. This amendment is accompanied by a floppy disk containing the sequences (SEQ ID NOs:1-133) in computer readable form, and a paper copy of the sequence information that has been printed from the floppy disk.

Also enclosed is a "Statement of Support Filing and Submission in Accordance with 37 C.F.R. §§ 1.1821-1.925", by James A. Coburn of Harbor Consulting, the preparer of the sequence listing, indicating that the information contained in the computer readable form (floppy disk) is identical to that of the paper copy.

This amendment contains no new matter. The amendments to the specification and/or claims are to provide a formal sequence listing and/or to provide appropriate cross-references to SEQ ID Numbers in accordance with 37 C.F.R. §§1.821 to 1.825. The sequence information provided herein finds support in the specification as filed.

Change in Correspondence Address.

A Revocation and Substitute Power of Attorney incorporating a change in correspondence address accompanies this document. In accordance with the instructions provided therein, please direct all future correspondence regarding the subject application to CUSTOMER NUMBER 22798, that is:



22798

PATENT TRADEMARK OFFICE

LAW OFFICES OF JONATHAN ALAN QUINE

P.O. BOX 458

Alameda, CA 94501

Tel: (510) 337-7871 / Fax: (510) 337-7877

If a telephone conference would expedite prosecution of this application, the Examiner is invited to telephone the undersigned at (510) 337-7871.

LAW OFFICES OF JONATHAN ALAN QUINE

P.O. BOX 458

Alameda, CA 94501

Tel: 510 337-7871

Fax: 510 337-7877

Respectfully submitted,

Tom Hunter

Attorney for Applicant(s)

Reg. No: 38,498

- Encl:
- 1) Copy of Notice to Comply With Requirement for Patent Applications Containing Nucleotide Sequence . . .
 - 2) Sequence listing paper copy and computer readable form (CRF)
 - 3) Petition for 1 month extension of time.



APPENDIX A

VERSION WITH MARKINGS TO SHOW CHANGES MADE IN 09/477,962 WITH ENTRY OF
THIS AMENDMENT

In the specification:

Page 3, lines 14-25:

In one embodiment, this invention provides an isolated nucleic acid comprising a nucleic acid selected from the group consisting of a nucleic acid encoding any one of Blm open reading frames (ORFs) 8 through 41, and/or a nucleic acid encoding a polypeptide encoded by any one of Blm open reading frames (ORFs) 8 through 41, and/or a nucleic acid amplified by polymerase chain reaction (PCR) using any one of the primer pairs identified in Table II and the nucleic acid of a bleomycin-producing organism as a template. The nucleic acid may comprise one or multiple (*e.g.* two, more preferably 3 or more) bleomycin open reading frames (*i.e.* *BLM* ORFs 8 through 41). One preferred nucleic acid comprises a nucleic acid encoding a C domain lacking one or more His residues of the conserved HHxxxDG (SEQ ID NO:4) active site for transpeptidation. In another preferred embodiment the nucleic acid comprises a nucleic acid encoding a protein encoded by a gene selected from the group consisting of *blmI*, *blmII*, and *blmXI*.

Page 15, lines 18-31:

Figure 8A shows a restriction map of the *blm* gene cluster from *Sv* ATCC15003 (B, *Bam*HI). 8B shows the relative position of the *blmI*, *blmII*, and *blmXI* genes to the two *blmAB* resistance genes (*blm^R*, Blm resistance). Individual open reading frames are represented by open arrows. Figure 8C (SEQ ID NO:127 & 128) shows the nucleotide sequence of the *blmI* gene. The potential ribosome-binding site (RBS) and the conserved motif for 4'-phosphopantetheinylation are underlined. The sequence has been deposited into GenBank under accession no. AF210249.

Figure 9 shows an amino acid sequence comparison of BlmI (SEQ ID NO:133) with PCP domains of known type I NRPSs (Grs-2 [P14688] (SEQ ID NO:129), 36% identity, 58% similarity; Srfa-3 [Q08787] (SEQ ID NO:130), 40% identity, 64% similarity; Vir-s [Y11547] (SEQ ID NO:131), 36% identity, 60% similarity; Saf-b [U24657] (SEQ ID NO:132), 40% identity, 54% similarity). Given in brackets are nucleotide sequence accession numbers. The shaded letters indicate similar amino acids.

Consensus residues are amino acids that are similar in more than three sequences. The signature motif for 4'-phosphopantetheinylation is underlined.

Page 68, line 8 through page 69, line 16:

The similarities among PPTases from different organisms are reduced to two short motifs separated by 40-45 residues: (V/I)G(V/I)D (SEQ ID NO:87), and (F/W)(S/C/T)XKE(A/S)hhK (SEQ ID NO:91) (Lambalot et al. *Chem. Biol.* (1996) 3:923-936; Walsh et al. *Curr. Opin. Chem. Biol.* (1997) 1:309-315). Our previous attempts to amplify PPTase sequences from *S. verticillus* chromosomal DNA using degenerate primers according to the two conserved motifs were unsuccessful (unpublished results), so we decided to narrow our target. PPTases have been classified in two groups, according to their specificity for the carrier-protein substrate: PPTases involved in polyketide/fatty acid biosynthesis use acyl carrier proteins (ACPs) as substrate, while those for non-ribosomal peptide biosynthesis use peptidyl carrier proteins (PCPs) or aryl carrier proteins (ArCPs) (Walsh et al. *Curr. Opin. Chem. Biol.* (1997) 1:309-315). Several "NRPS-type" PPTase sequences were used to screen the databases to look for actinomycete homologues, and four proteins of unknown function were found: NshC from *Streptomyces actuosus* (Li et al. *Gene* (1990) 91:9-17), SC5A7.23 from *S. coelicolor* (GenBank AL031107), an unnamed protein from *Streptomyces* sp. strain TH1 (Mori et al. *J. Bacteriol.* (1997) 179:5677-5683), and Rv2794c (later renamed PptT (Quadri et al. *Chem. Biol.* (1998) 5:631-645)) from *Mycobacterium tuberculosis* (GenBank AL008967). The alignment of the actinomycete sequences showed the two motifs conserved in all PPTases and an additional motif - the "THC" motif: PXWPGX₂GS(M/L)THCXGY (SEQ ID NO:86), located about 15 amino acids upstream of the (V/I)G(V/I)D motif (SEQ ID NO:87). The "THC" motif is not universally conserved in all PPTases, but it can be detected also in some non-actinomycete PPTases like EntD (Coderre et al. *J. Gen. Microbiol.* (1989) 135:3043-3055). Using a recently developed method of PCR primer design (the CODEHOP strategy (Consensus-DEgenerate Hybrid Oligonucleotide Primer) (Rose et al. *Nucleic Acids Res.* (1998) 26:1628-1635), two primers were designed around the typical C-terminal PPTase motif (primers KEA-1: 5'-T GCA GCA GAA CAG GAG GCK NYC CCA NKG-3' (SEQ ID NO:88) and KEA-2: 5'-TG GGT CAG CGG GTA CCA NRC YTT RWA-3' (SEQ ID NO: 89, H=C+A, N=A+C+T+G, Y=C+T, K=G+T, R=A+G, W=T+A)), and one primer was designed from the "THC" motif (primer THC: 5'-C GGC ATG GTC GGC TCC HTN ACN CAY TG-3', SEQ ID NO:90, H=C+A, N=A+C+T+G, Y=C+T, K=G+T, R=A+G, W=T+A); this motif is not universally conserved in PPTases of all organisms). Using *S. verticillus* chromosomal DNA as template,

no amplification product was detected using the THC and the KEA-1 primers. The set of primers THC/KEA-2 successfully amplified a single band of the expected size (about 250 bp), which was gel-purified and cloned. Eight individual clones were sequenced, and all of them resulted to be identical (except differences due to primer utilization) and highly similar to the putative actinomycete PPTases. The PCR fragment was used as a probe to screen a *S. verticillus* genomic library by colony hybridization. Of the 10,000 colonies screened, 25 positive clones were identified, and then confirmed by Southern analysis to contain the same 4.6-kb *Bam*HI hybridizing band. The 4.6-kb DNA fragment was subcloned, and the nucleotide sequence of a 1,761-bp *Bam*HI-*Sal*I region was determined (SEQ ID NO. 3).

Page 69, line 17 through page 70, line 20:

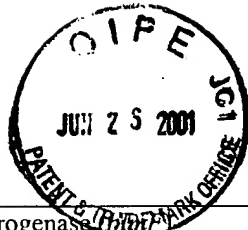
The sequence of the 1,761-bp *Bam*HI-*Sal*I fragment was analyzed for coding regions by using the CODONPREFERENCE and TESTCODE programs of the GCG package (Genetics Computer Group, Madison, Wisconsin). Two complete ORFs (*pptA*, *orf3*) and two incomplete ORFs (*orf1*, *orf4*) were identified within the sequenced region (Figure 13). The first ORF from left to right (designated *orf1*) starts out of the analyzed area and ends with a TGA codon at position 248 of the sequenced fragment. Comparison of the deduced product of *orf1* with proteins in databases showed similarities with Rv2795c from *Mycobacterium tuberculosis* (GenBank AL008967) and SC5A7.22 from *S. coelicolor* (GenBank AL031107), both of unknown function. The second ORF, *pptA*, contains the sequence amplified by PCR and used for the cloning of this locus. It comprises 741 nucleotides, starting with a GTG codon (position 245) which is coupled to the stop codon of *orf1*, and ending with a TAA codon. The starting codon of *pptA* is preceded by a potential ribosomal binding site (RBS), GGGAG. The overall (76.6%) and third codon position (93.9%) G+C contents and the codon usage of *pptA* are similar to those found in other *Streptomyces* genes, with the exception of the stop codon (TAA), which is most uncommon in this group of organisms (Wright et al. *Gene* (1992) 113:55-65). The *pptA* gene encodes a protein of 246 amino acids with a predicted molecular mass of 25,619 Da and a pI of 4.76, which contains the conserved PPTase motifs. Databases searches with PptA showed significant similarities to the putative actinomycete PPTases (39-52%/48-61% identity/similarity) and to confirmed bacterial PPTases such as EntD from *E. coli* (17%/24% identity/similarity) (Lambalot et al. *Chem. Biol.* (1996) 3:923-936). The third ORF, *orf3*, is separated from *pptA* by an apparently noncoding DNA region of 153 bp, and it is transcribed in opposite and convergent direction with respect to *orf1-pptA*. The gene *orf3* comprises 240 nucleotides, starting with an ATG codon (position 1358) and ending with TGA. The starting codon of *orf3* is preceded by the

sequence GAAGG, a potential RBS. The deduced product of *orf3* encodes a protein of 79 amino acids with a predicted mass of 7,555 Da and a pI of 7.17. The Orf3 protein shows similarities to the N-terminal region of SC5H1.35c, a protein of unknown function from *S. coelicolor* (GenBank AL049863). Analysis of Orf3 with the SignalP program (Nielsen et al. *Protein Engineer.* (1997) 10:1-6) predicts an N-terminal signal peptide which would be cleaved between residues 27 and 28 (ALA-DS), suggesting that the mature protein (52 amino acids, 5,099 Da, pI 4.31) would be secreted. Between *orf3* and *orf4* there is an apparently noncoding region of 251 nucleotides. The *orf4* gene is transcribed in opposite and divergent direction with respect to *orf3*. It starts with an ATG codon at position 1610, preceded by a potential RBS (GGAGG), and ends out of the sequenced fragment. The deduced protein product (50 amino acids) of the incomplete *orf4* contains a potential NAD/FAD binding motif, GXGX₂GX₃GX₆G (SEQ ID NO:92) (Scrutton et al. *Nature* (1990) 343:38-43), showing low similarities to diverse oxidoreductases.

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Table II. *Blm* gene cluster open reading frames (ORFs) and primers for ORF amplification.

Orf #	Position	Activity	Method	Primers		Seq ID No.
				Forward	Reverse	
orf-8 SEQ ID NO:115	76183-77457	Oxygen-independent coproporphyrinogen III oxidase	Gapped-blast comparison ¹	F: ATGAGCCACGCCATCGGA R: TCAGGCGCGTTCGGGGGC		5 6
orf-9 SEQ ID NO:114	74690-76186	ADP-heptose synthase (<i>blmC</i>)	Gapped-blast comparison ¹	F: GTGAACACCGACCTGCCC R: TCATGGGGTGTCTCCCTC		7 8
orf-10 SEQ ID NO:113	74421-74693	Peptidyl carrier protein (<i>blmI</i>)	Expression and biochemical characterization. ²	F: ATGAGCGCCCCGCGGGGC R: TCACCGGTCCCGCTCCCC		9 10
orf-11 SEQ ID NO:112	72787-74424	Carbamyltransferase (<i>blmD</i>)	Gapped-blast comparison ¹	F: ATGAGCGCCGACCCGTCC R: TCATGAGCGGGCCGCCGT		11 12
orf-12 SEQ ID NO:111	71618-72790	ADP-heptose:LPS heptosyl transferase (<i>blmE</i>)	Gapped-blast comparison ¹	F: ATGACCACCCCATGACC R: TCATGGGGTACTCCTGAT		13 14
orf-13 SEQ ID NO:110	70983-71546	Homolog of mbtH in the synthesis of mycobactin	Gapped-blast comparison ¹	F: ATGACCACGACCCGCGG R: TCAGGTGCCGGACACGCG		15 16
orf-14 SEQ ID NO:109	69598-70986	Peptide synthetase (condensation, <i>blmII</i>)	Gapped-blast comparison ¹	F: GTGACCGCCCCCGGCACA R: TCATCGGTGGCTCCTCGT		17 18
orf-15 SEQ ID NO:108	68582-69601	Regulatory gene (homolog of <i>syrP</i>)	Gapped-blast comparison ¹	F: GTGAACCGGCACGGCCCC R: TCACGCGCTCACCTCGTC		19 20
orf-16 SEQ ID NO:107	65778-68585	Mutated peptide synthetase-oxidase (NRPS-0, <i>blmIII</i>)	Gapped-blast comparison ¹	F: GTGACGAGCGCCCGGCCC R: TCACGGGGCCTCCGTGCG		21 22
orf-17 SEQ ID NO:106	57901-65781	Peptide synthetase (NRPS-2-1, <i>blmIV</i>)	Expression and biochemical characterization. ²	F: ATGCTGCACGGCGCCGCG R: TCACTCCGGTCCACCTCC		23 24
orf-18 SEQ ID NO:105	55899-57815	Asparagine synthetase	Gapped-blast comparison ¹	F: GTGAGGCCCGTGTGCGGC R: TCAGCCACCGTTGCCGCC		25 26
orf-19	54418-	Homolog of hydroxylase-	Gapped-blast	F: GTGAAGGACCTCGGCCGG		27



SEQ ID NO:104	55902	dehydrogenase (blmI)	comparison ¹	R: TCACTCCCCCGGTGCCGG	28
orf-20	53427-	Nucleotide-sugar epimerase	Gapped-blast	F: GTGACATGGACCGTGGTG	29
SEQ ID NO:103	54404	(blmG)	comparison ¹	R: TCAGGCATCGGCCCTCCC	30
orf-21	51493-	Peptide synthetase	Gapped-blast	F: ATGCGCGGGCATGACGAC	31
SEQ ID NO:102	53430	(NRPS-3CT, blmV)	comparison ¹	R: TCACGGTGTCTCTCCCTC	32
orf-22	43263-	Peptide synthetase	Expression and	F: ATGAGCCGGCCGGCCGGC	33
SEQ ID NO:101	51290	(NRPS-5-4-3, blmVI)	biochemical characterization. ²	R: TCATGCTCGGTTCATCGCC	34
orf-23	39610-	Peptide synthetase	Expression and	F: GTGACCACGCCCCGCATC	35
SEQ ID NO:100	43266	(NRPS-6, blmVII)	biochemical characterization. ²	R: TCATTCCGGACGCGGGCA	36
orf-24	34088-	Polyketide synthase	Gapped-blast	F: ATGAGCCATGCCGACGCG	37
SEQ ID NO:99	39613	(blmVIII)	comparison ¹	R: TCACAGCACCACCTCTTC	38
orf-25	30891-	Peptide synthetase	Gapped-blast	F: ATGACCCCGGCCGCCGAC	39
SEQ ID NO:98	34091	(NRPS-7, blmIX)	comparison ¹	R: TCATCGTCCGCCGCCCTTT	40
orf-26	24406-	Peptide synthetase	Gapped-blast	F: ATGCCTCGGTGTGCCCGA	41
SEQ ID NO:97	30894	(NRPS-9-8, blmX)	comparison ¹	R: TCATTCCGCGGCACCTCC	42
orf-27	22127-	Peptide synthetase	Gapped-blast	F: GTGGGTTTCCGTCGAGCG	43
SEQ ID NO:96	24193	(condensation, blmXI)	comparison ¹	R: TTACACCTCCGTTTCTC	44
orf-28	21367-	Phosphatidylserine	Gapped-blast	F: ATGGCACAGGACCTGAAC	45
SEQ ID NO:95	22086	decarboxylase	comparison ¹	R: TCAACGCCACCGGATCTT	46
orf-29	19161-	Transmembrane transporter	Gapped-blast	F: GTGAGCTCCCTCGCCGTC	47
SEQ ID NO:94	20909		comparison ¹	R: TCATCGTCGGGCACTCGG	48
orf-30	18823-	Metal dependent regulatory	Gapped-blast	F: GTGCCGGTTCCGCTGTAT	49
SEQ ID NO:93	19164	element	comparison ¹	R: TCACCGGGCACTGACCTC	50
orf-31	18660-	PHNA homolog	Gapped-blast	F: GTGACCAGAACCTTCCG	51
SEQ ID NO:116	18307		comparison ¹	R: TCAGACCTTCTTGACCAC	52
orf-32	17736-	Peptide synthetase	Gapped-blast	F: ATGGCCTCAGACGCTTTG	53
SEQ ID NO:117	9211	(NRPS-11-10)	comparison ¹	R: TCATTGAGACTCCTCCTC	54
orf-33	9214-	Putative transporter	Gapped-blast	F: ATGATGAAGTCAAGCCGC	55
SEQ ID NO:118	7859		comparison ¹	R: TCAGTGGCTTACAAGGAG	56
orf-34	7797-	Homolog of clavaminic	Gapped-blast	F: ATGACTGACCTGCCGTTG	57
SEQ ID NO:119	6784	acid synthase	comparison ¹	R: TCACACCAGCAGCGAGGT	58
orf-35	6773-	Thioesterase	Gapped-blast	F: ATGGATTTCCTCCCTCACC	59
SEQ ID NO:120	6021		comparison ¹	R: TCATGCCCCCTACCTCGGC	60
orf-36	6024-	Putative transporter	Gapped-blast	F: ATGACCGCGCGCTCGAC	61
SEQ ID NO:121	4741		comparison ¹	R: TCACTCCTCGGCTTCGGC	62
orf-37	4733-	Unknown	Gapped-blast	F: GTGTCCAAGAACGCGCGG	63
SEQ ID NO:122	3915		comparison ¹	R: TCATCGGCTCGCTCGTG	64
orf-38	3918-	Peptide synthetase	Gapped-blast	F: ATGACCCTCACCCTGCGG	65
SEQ ID NO:123	2182	(NRPS-12)	comparison ¹	R: TCACTCGGGCACTCCTTC	66
orf-39	2185-	Regulatory gene	Gapped-blast	F: GTGACCGGTTCCGTAACG	67
SEQ ID NO:124	1199	(homolog of <i>SyrP</i>)	comparison ¹	R: TCATGAGTCCGCCGAGGT	68
orf-40	1015-1	Peptide synthetase	Gapped-blast	F: ATGACAGAGGTCCGAGGT	69
SEQ ID NO:125			comparison ¹	R: CCCGGCAACCGCCCTCCC	70
orf-41	On a	4'-phosphopantetheinyl	Expression and	F: GTGATCGCCGCCCTCCTG	71
SEQ ID NO:126	separate	transferase (<i>pptA</i>)	biochemical characterization. ²	R: TTACGGGACGGCGGTCCG	72
	sequence				



In the claims:

4. The isolated nucleic acid of claim 1, wherein said nucleic acid comprises a nucleic acid encoding a C domain lacking one or more His residues of the conserved HHxxxDG (SEQ ID NO:4) active site for transpeptidation.

28. The polypeptide claim 25, wherein said polypeptide comprises a C domain lacking one or more His residues of the conserved HHxxxDG (SEQ ID NO:4) active site for transpeptidation.